

# ELYRA – 3D PALM Experimental PSF Multichannel Alignment



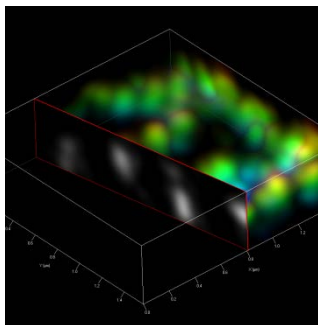
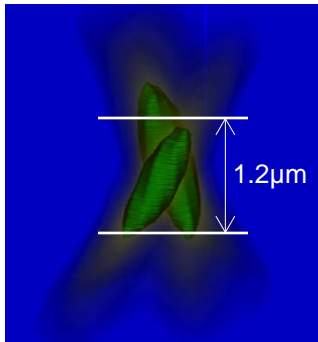
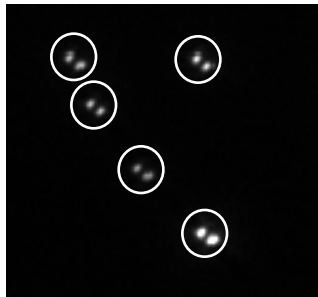
Renée Dalrymple

Superresolution Applications Specialist

October 2, 2015

# Outline

## Workflow for 3D PALM on ELYRA P.1 or PS.1



1 Acquire PSF



2 Experimental PSF



Automatic

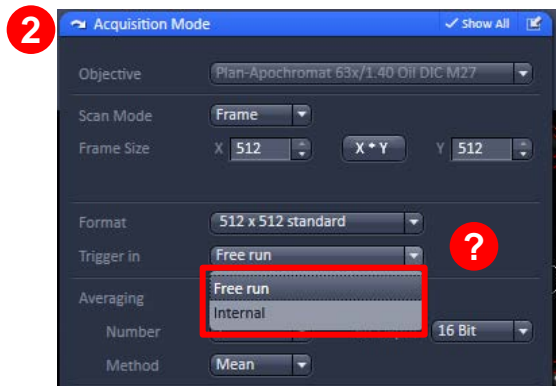
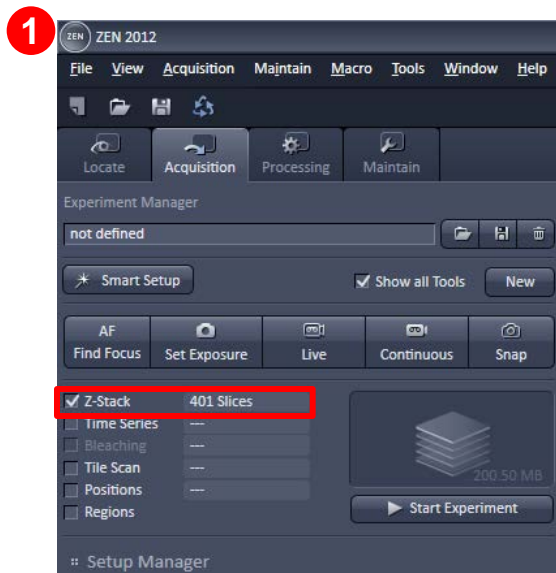
3 Create Localization LUT



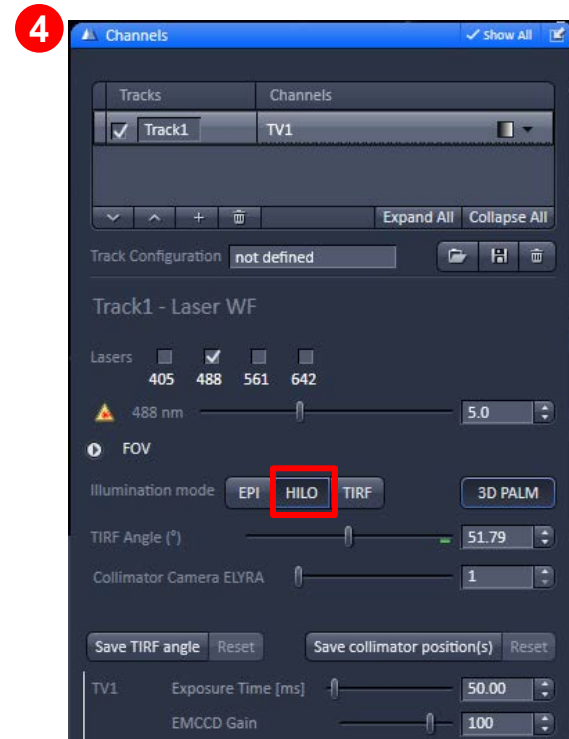
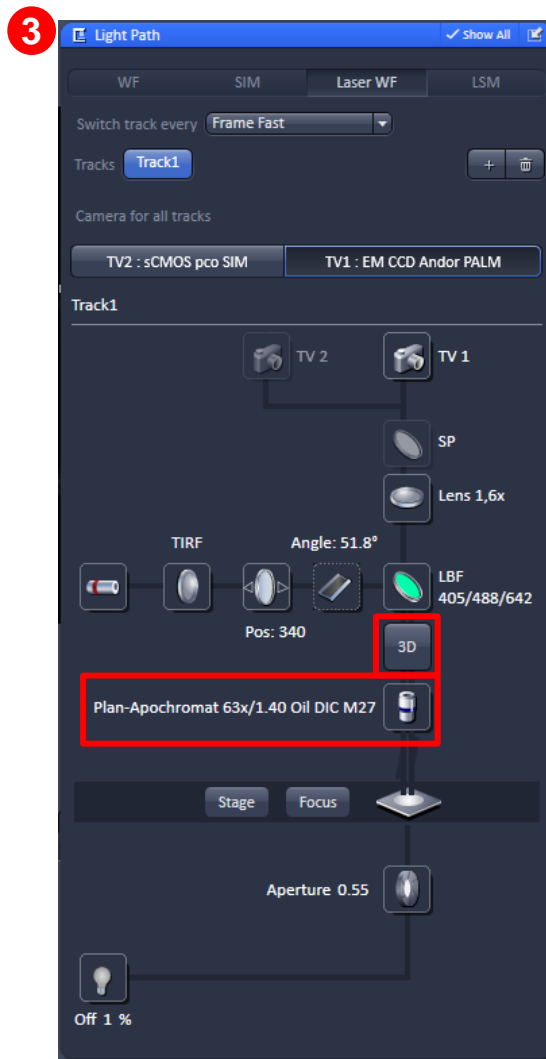
4 Apply to Images

# 1. Acquire PSF

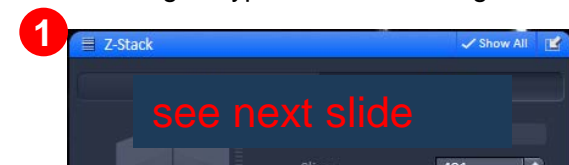
Using (100 nm tetraspec) beads, 3D slider in and 4  $\mu\text{m}$  range



internal: recommended  
free run: works



HILO: recommended  
time / gain typical: 50 ms / 100 gain



z-stack settings: see next slide

# 1. Acquire PSF

*Z-stack definition*



Z-Stack

First/Last Center

Range 4.00  $\mu\text{m}$

Slices 401

Interval 0.010

Smallest 0.000  $\mu\text{m}$

Keep  Interval  Slice

Center 0.00

Offset 0.00

Use Piezo

Position ( $\mu\text{m}$ ) 0.00

Optimize Sectioning and Step

Correction

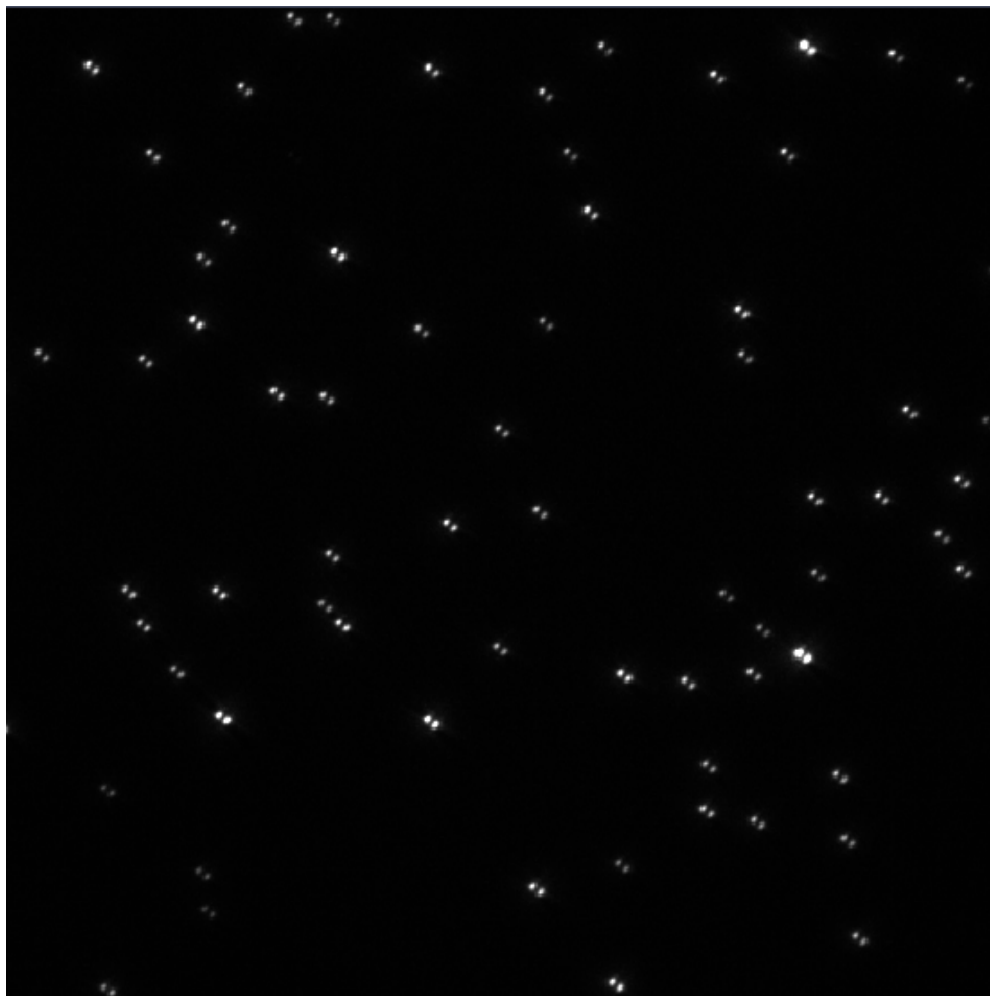
**Use 4  $\mu\text{m}$  range**  
to avoid artefacts

**10 nm step size**  
for good oversampling

**Use piezo**  
make sure this is active

# 1. A typically suitable z-stack

*Sparse beads, well separated lobes, in dynamic range*



## Rules of Thumb:

### (1) approx. 10 - 80 beads in FOV

If fewer: poor statistics

If too crowded: too many beads discarded

**Remedy:** Look for region in sample with appropriate density

### (2) Exploit full dynamic range

Avoid too bright bead agglomerates in FOV

### (3) Lobes well separated

Check oil (bubbles)

Check 3D slider (Maintain tab)

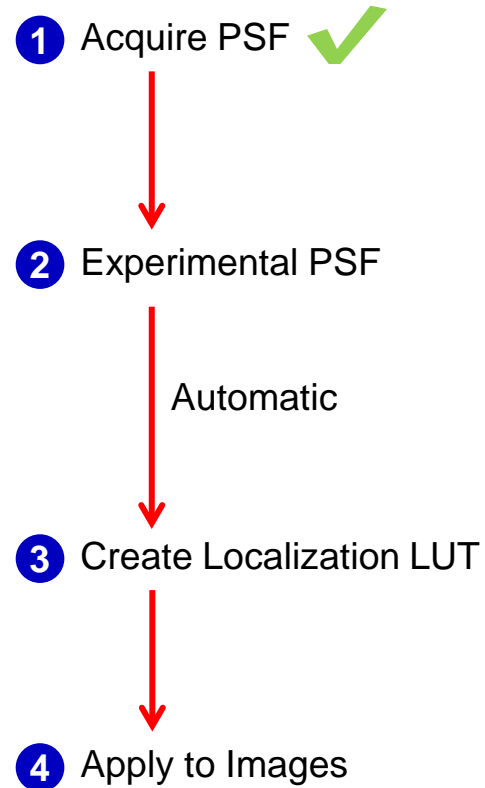
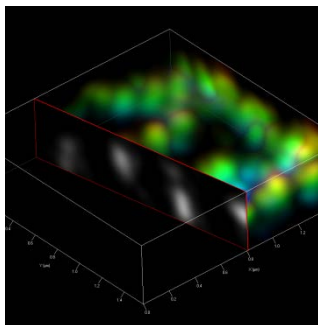
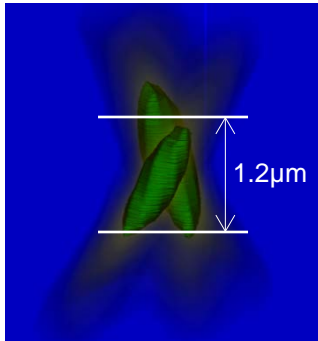
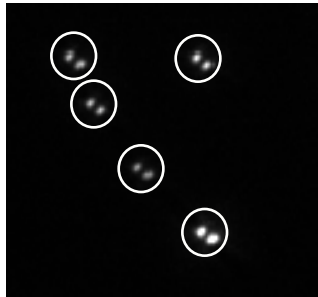
Check collimator position

Vary HILO angle

Use other objective

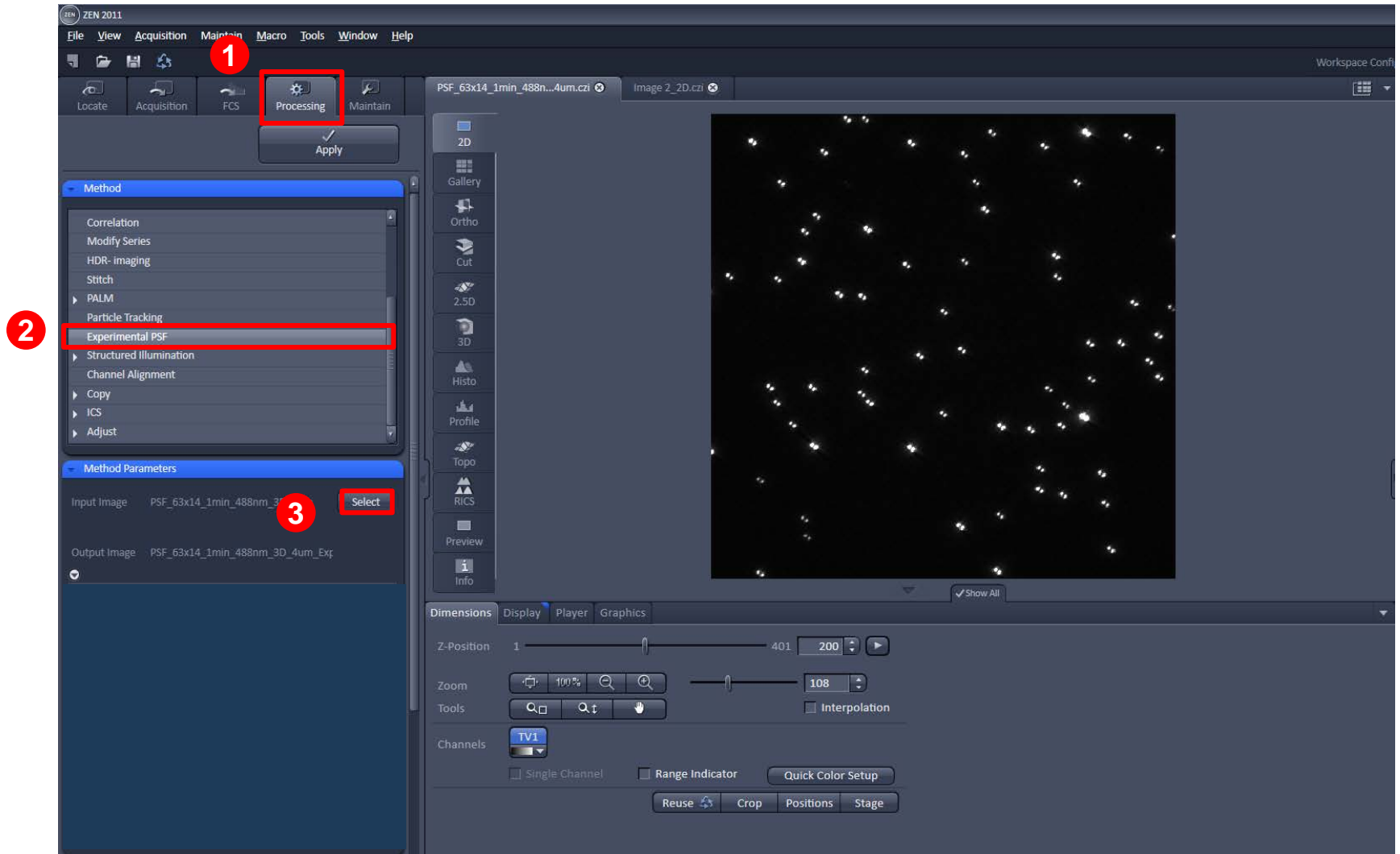
# Outline

## Workflow for 3D PALM on ELYRA P.1 or PS.1



# 2. Build Experimental PSF

*Processing Tab, Experimental PSF, Select*



# 2. Build Experimental PSF

*Automatically recognizes 3D-PALM situation*



The image shows the ZEN 2011 software interface. The top menu bar includes File, View, Acquisition, Maintain, Macro, Tools, Window, and Help. Below the menu is a toolbar with icons for Locate, Acquisition, FCS, Processing, and Maintain. The Processing tab is active, and the 'Apply' button is highlighted with a red box and a '5' in a red circle. The left sidebar contains a 'Method' list with 'Experimental PSF' selected, and 'Method Parameters' with 'Input Image' and 'Output Image' fields. The 'Automatic' dropdown menu is highlighted with a red box and a '4' in a red circle. The main image area shows a 2D view of particles on the left and a 3D view of particles on the right, both with red circles around them. A red exclamation mark is in the top right corner. The bottom panel shows 'Dimensions' with 'Z-Position' set to 200, 'Zoom' at 103, and 'Channels' set to TV1.



## 2. Build Experimental PSF

*Bead selection display in preview*



ZEN will only use well-behaved patterns:

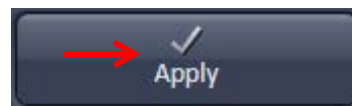
**White circles** show which patterns are used

**Red circles** show the discarded patterns

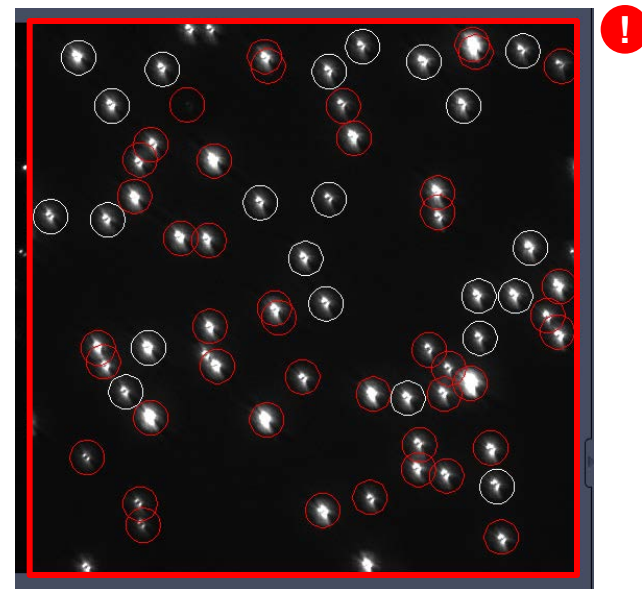
Discarding may be due to:

- (a) Overlap between patterns
- (b) SNR too low
- (c) Pattern too close to edge of image

**Possibilities**



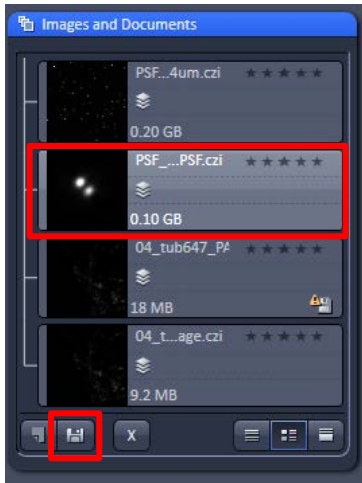
- (1) Accept auto settings as are and go (Apply)
- (2) Inspect selection closer (by moving slider)
- (3) Refine / Modify selection criteria (manual)



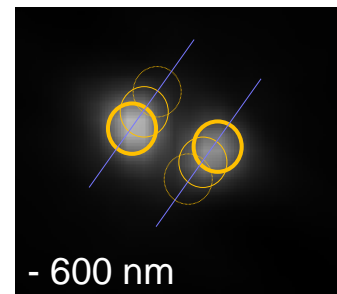
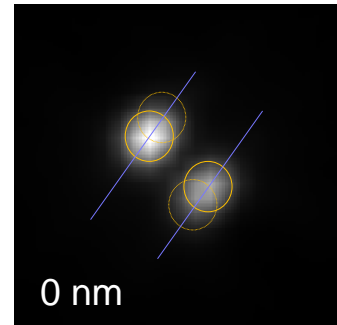
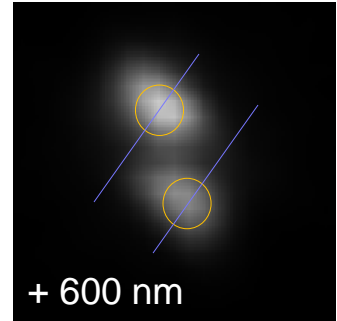
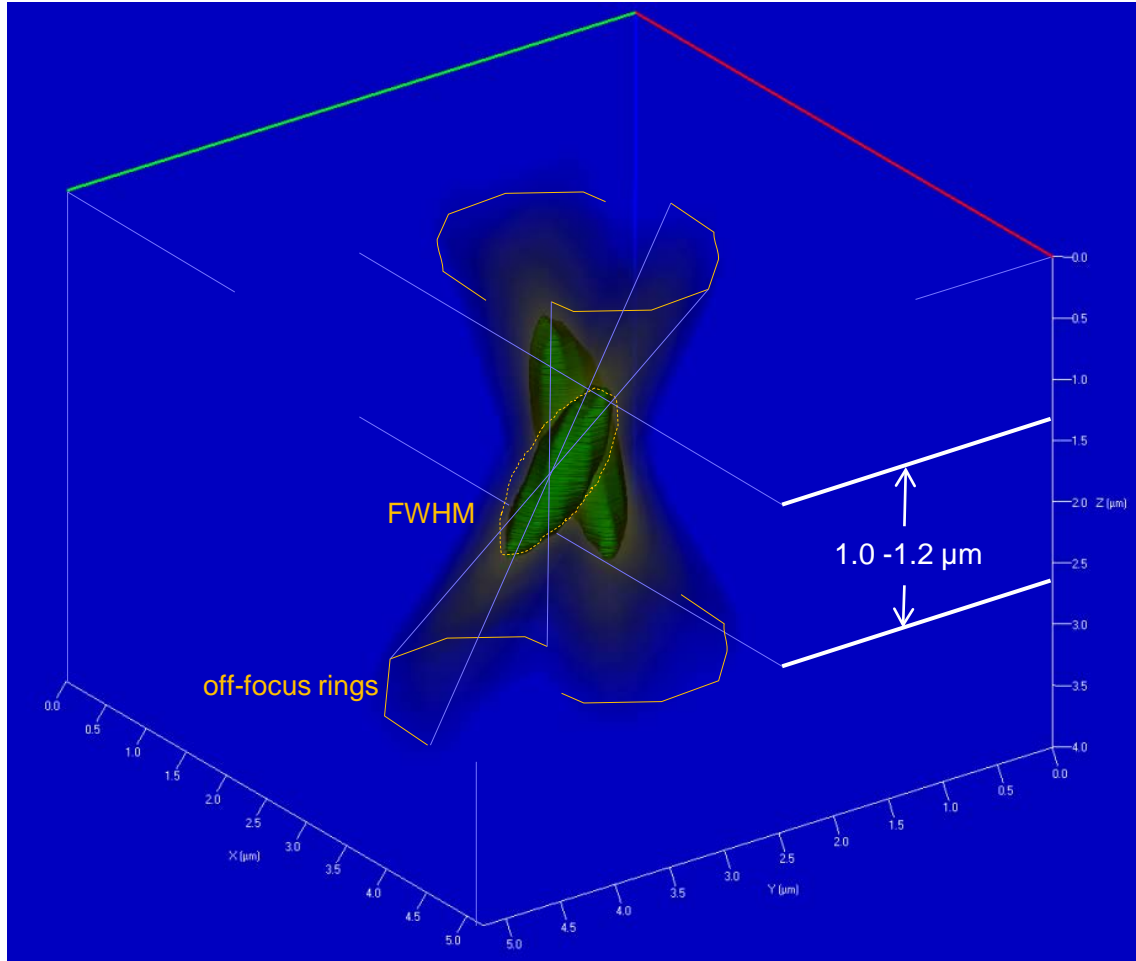
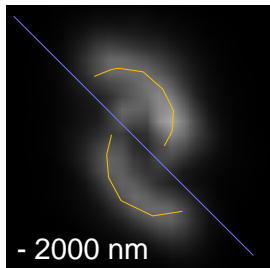
approx. 60 total  
approx. 20 selected

# 2. Build Experimental PSF

*PSF extends over several microns*

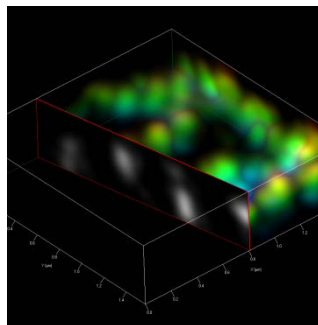
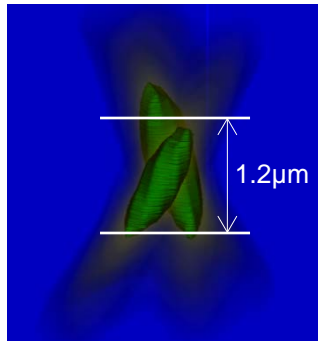
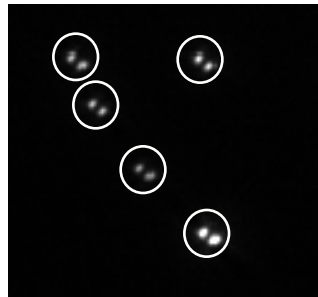


**SAVE!**



# Outline

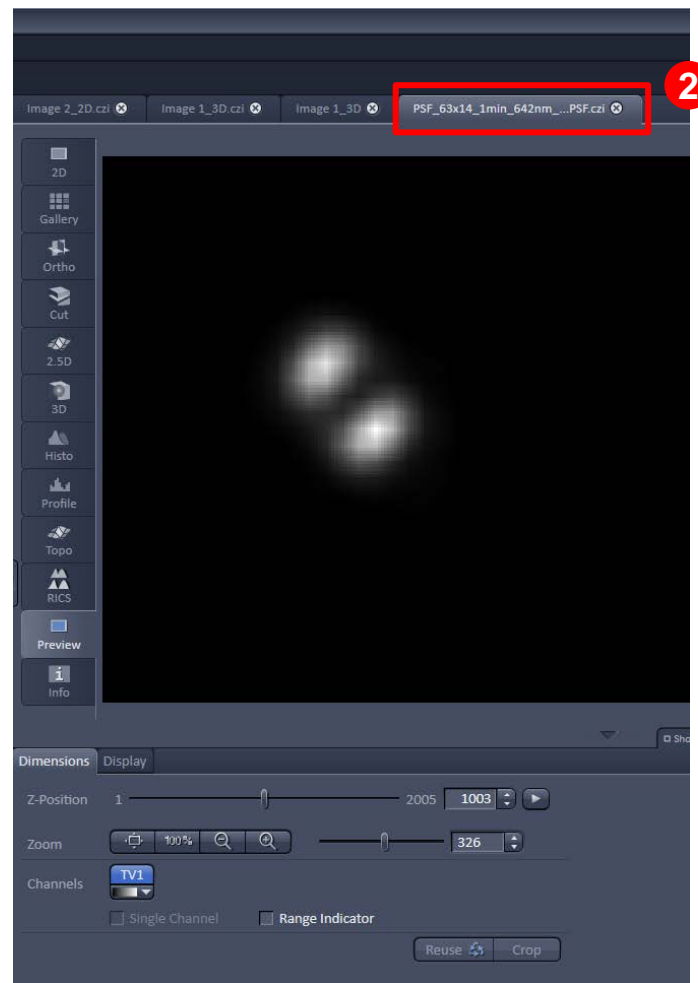
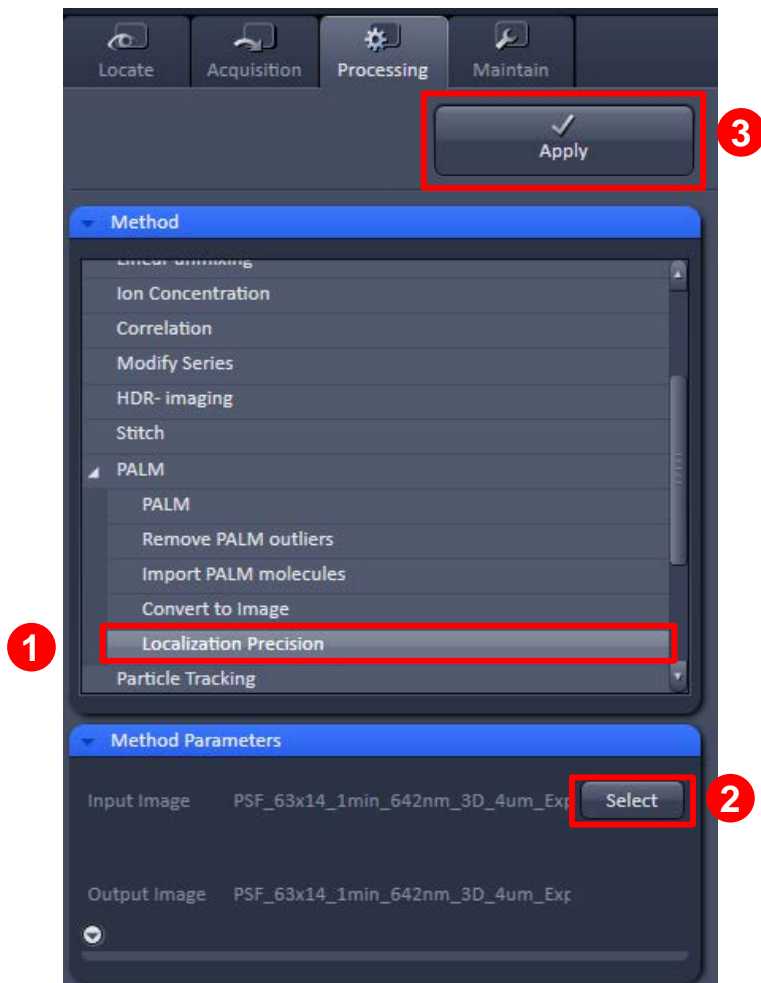
## Workflow for 3D PALM on ELYRA P.1 or PS.1



- 1 Acquire PSF ✓
- 2 Experimental PSF ✓  
Automatic
- 3 Create Localization LUT
- 4 Apply to Images

# 3. Create Localization Precision LUT

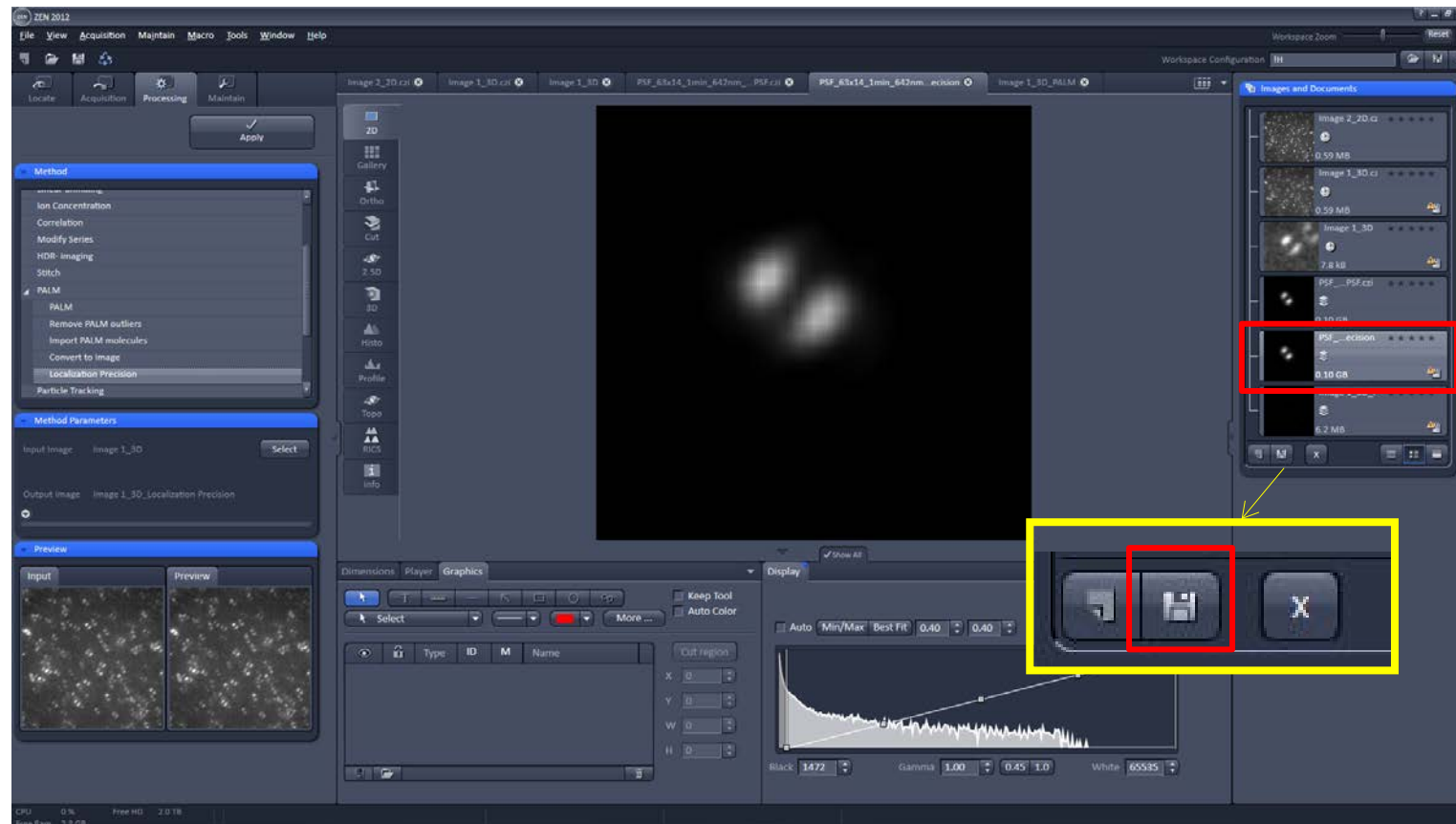
Localization Precision, Select Experimental PSF File, Apply.



! Takes a few minutes!

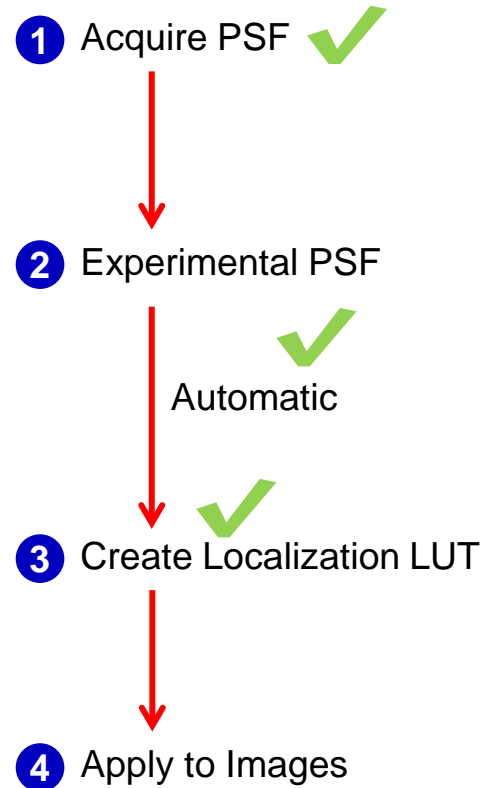
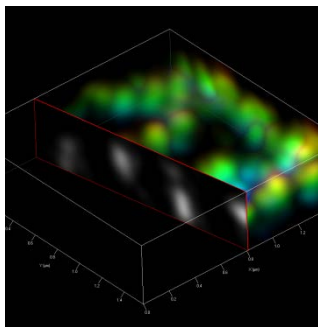
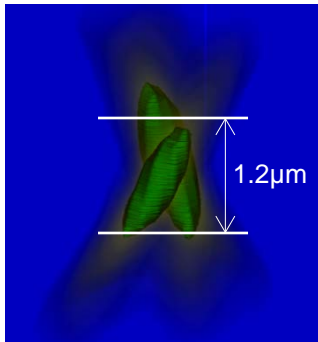
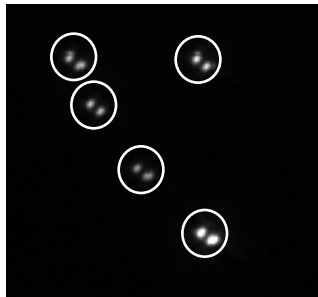
# 3. Create Localization Precision LUT

*After it is done: Just save.*



# Outline

## Workflow for 3D PALM on ELYRA P.1 or PS.1



# 4. Apply PSF File to images

*Load sequence, assign proper Precision LUT and go.*



(1) Processing -> PALM

(2) select image

(!) select LUT: this is your experimental PSF you just created

(?) choose:

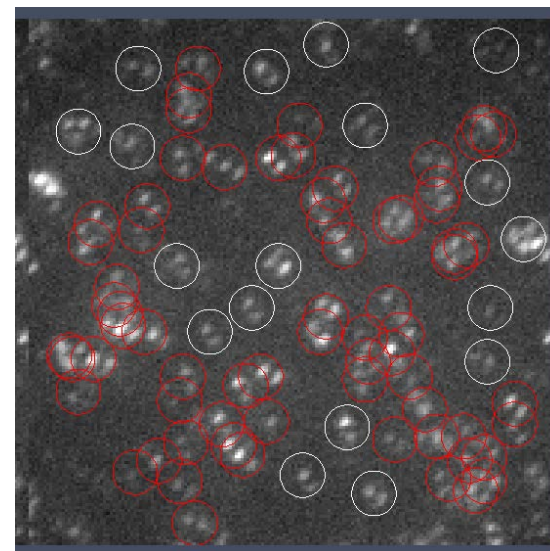
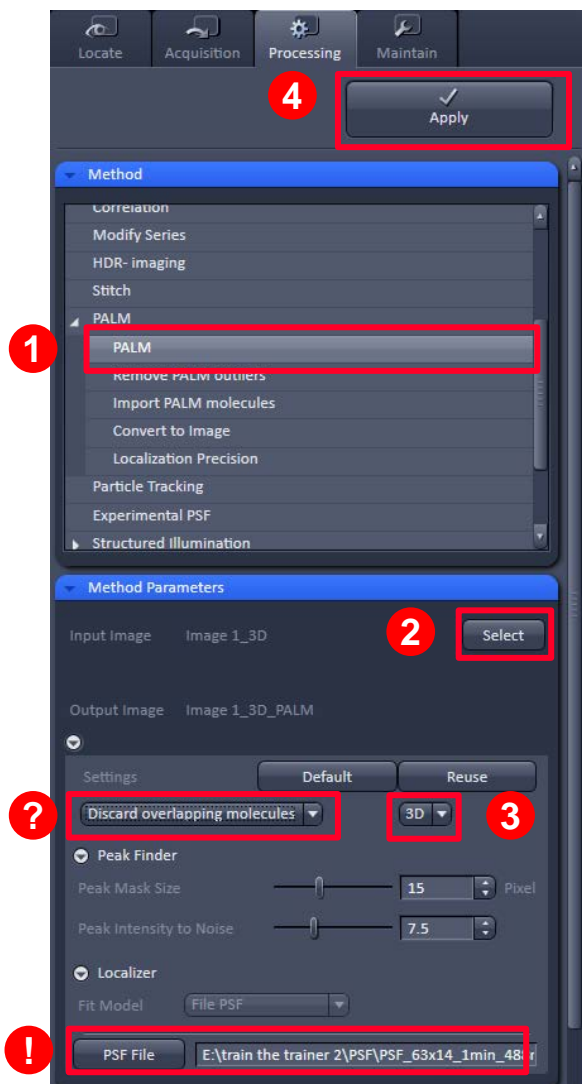
(a) account for overlap  
(slow, more patterns)

(b) discard overlap  
(fast, lots of discarding)

(c) ignore overlap  
(more errors)

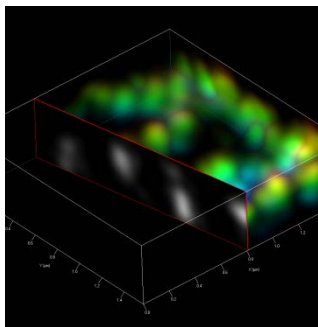
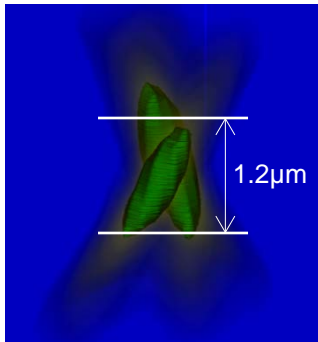
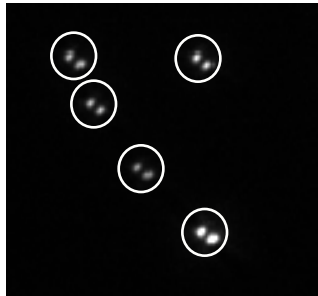
(3) 3D  
(automatically selected)

(4) Apply



# Outline

## Workflow for 3D PALM on ELYRA P.1 or PS.1



- 1 Acquire PSF ✓
- 2 Experimental PSF  
Automatic ✓
- 3 Create Localization LUT ✓
- 4 Apply to Images ✓



## Procedure:

Requirement: There must be fiducials present in the images of both channels.

- 1) After processing the raw data, if both channels were acquired together separate each color as its own PALM file.
  - a. Perform fiducial drift correction on each channel image
  - b. Write down the x, y, z positions of the common fiducials between each color
  - c. Render the images with the desired lateral and axial resolution. Be sure to check that both colors have exactly the same pixel size and z-plane thickness.
  - d. Convert each channel to an image
  
- 2) Combine the two “convert to image” files into one image using the Channel Alignment function
  - a. Select the two colors into “input 1” and “input 2”, check “input 2” box, uncheck “Fit” and apply.
  - b. Note: the data should be simply combined and not shifted/corrected.
  - c. Note: The file size of the combined image must be less than 4 GB.

# 3D PALM Multicolor Channel Alignment



- 3) Use the Channel Alignment function again on the resulting image from step 2.
  - a. Select the result from step 2 above into “input 1”
  - b. Uncheck “input2”, uncheck “fit”
  - c. Based on the x,y,z positions of the corrected fiducials you wrote in step **1b** above, calculate the average pixel shifts required for the channel alignment function:
    - i. Fiducial\_A = (Xa, Ya, Za), and Fiducial\_B = (Xb, Yb, Zb)
    - ii. Shift coefficient X =  $Xa - Xb / \text{pixel size}$
    - iii. Shift coefficient Y =  $Ya - Yb / \text{pixel size}$
    - iv. Shift coefficient Z =  $Za - Zb / \text{zSliceThickness}$
  - d. Using only “LATERAL” alignment, manually enter the XYZ coefficients above into the channel alignment function.
    - i. Make sure the channels are properly matched ( ID = 0 is matched to first channel, ID = 1 matched to second channel)
  - e. Hit Apply

Note 1: Consider the sign of the coefficients to make sure they are not flipped.

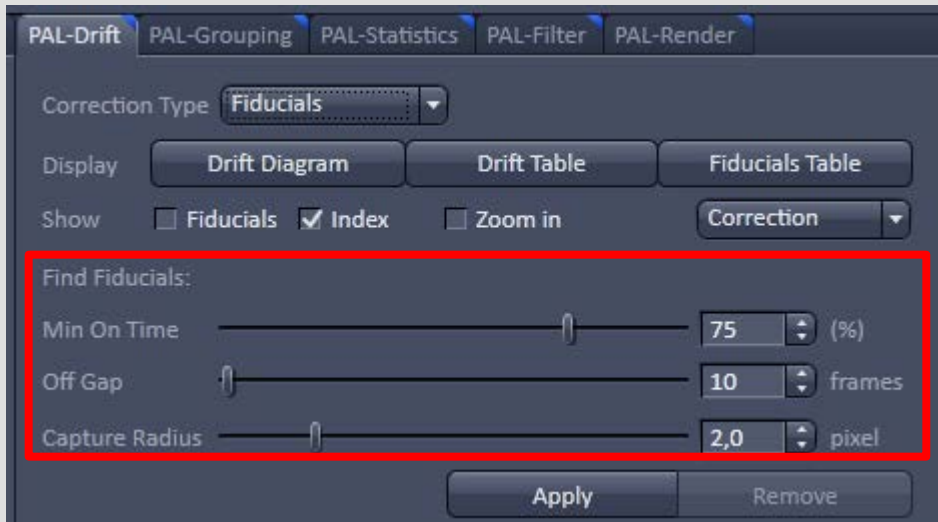
Note 2: The quality of the color fits depend critically on the quality of the fiducial drift correction. If you have poor fiducials or very few (2 or less), then the alignment may not be accurate.

# 3D PALM Multicolor Channel Alignment



## 1a. Drift Correct using Fiducials

Automatically detect fiducials:



Definition to auto-find fiducials.  
Fiducials can also be hand-picked

Hand-picking fiducials:  
see next slide

Camera pixel corresponds to 100nm  
(e.g. Capture Radius 2,0 = 200 nm)

# 3D PALM Multicolor Channel Alignment



## 1a. Drift Correct using Fiducials

Hand select fiducials:

The screenshot displays the PALM software interface. At the top, a table lists fiducial data:

Use	Index	X [μm]	Y [μm]
<input checked="" type="checkbox"/>	1	0.86	0.89

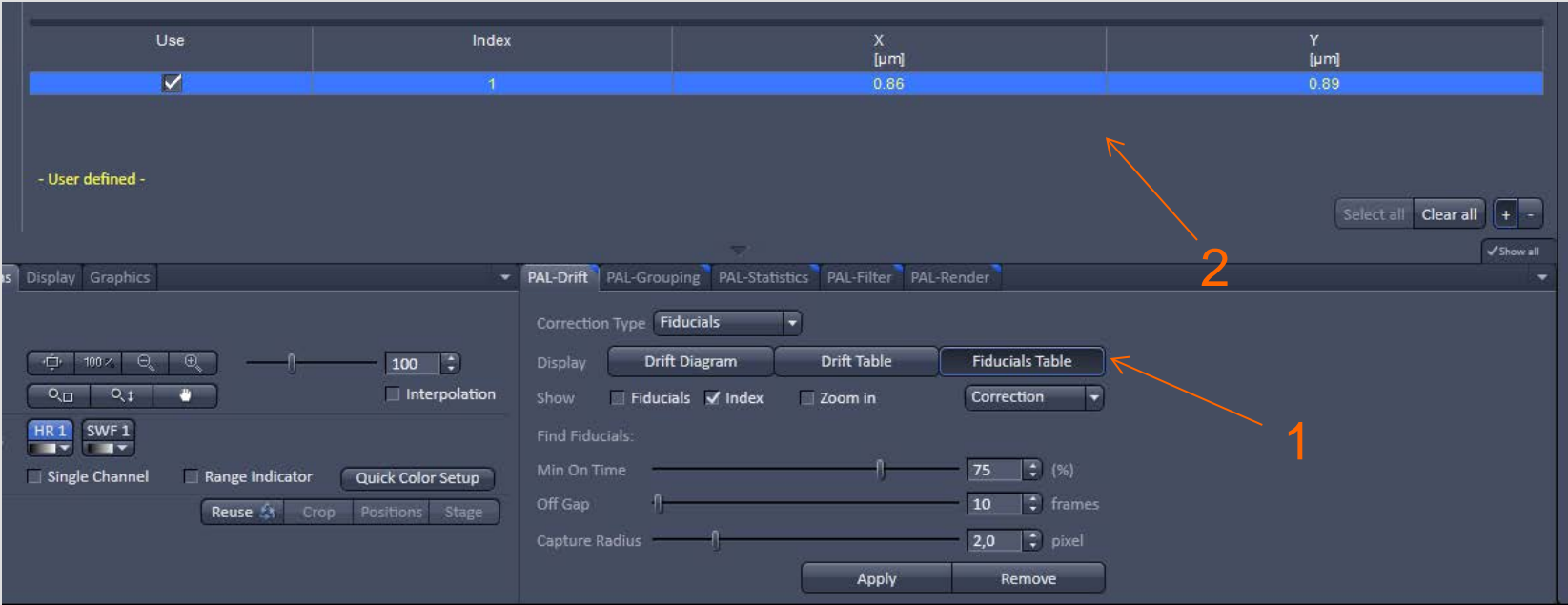
Below the table, the text "- User defined -" is visible. To the right of the table are buttons for "Select all", "Clear all", and a "+" button. An orange arrow labeled "2" points to the "+" button.

The main interface shows the "PAL-Drift" tab selected. The "Correction Type" is set to "Fiducials". The "Display" section has "Fiducials Table" selected. The "Show" section has "Index" checked. The "Find Fiducials" section has "Min On Time" set to 75%, "Off Gap" set to 10 frames, and "Capture Radius" set to 2.0 pixels. An orange arrow labeled "1" points to the "Fiducials Table" button.

1. Select Fiducials Table
2. Click on (+) then hover over in image and click to select fiducial

# 3D PALM Multicolor Channel Alignment

1b. Write down x, y, z, positions of fiducials



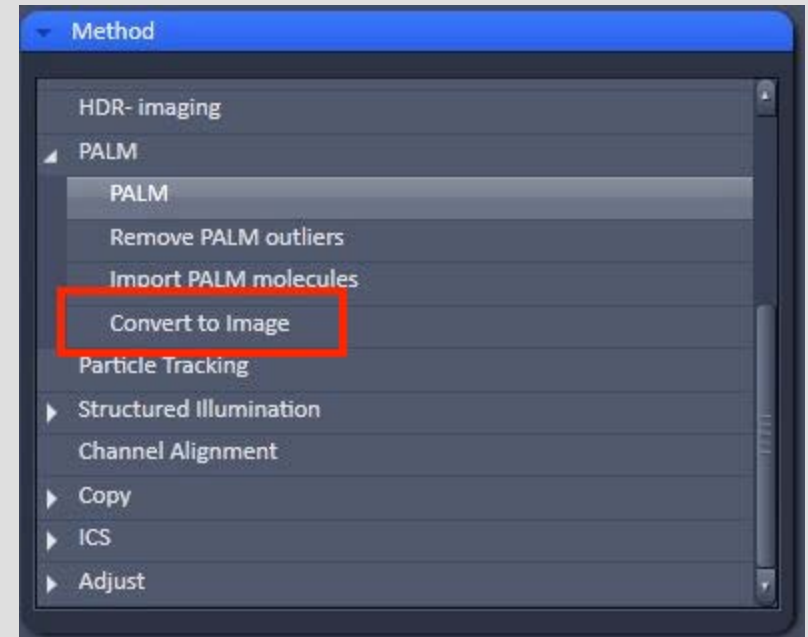
1. Select Fiducials Table
2. Write down the x, y, z positions of the common fiducials between each color

# 3D PALM Multicolor Channel Alignment

*1c-d. Render and convert to images*



- c. Render the images with the desired lateral and axial resolution. Be sure to check that both colors have exactly the same pixel size and z-plane thickness.
- d. Convert each channel to an image



# 3D PALM Multicolor Channel Alignment

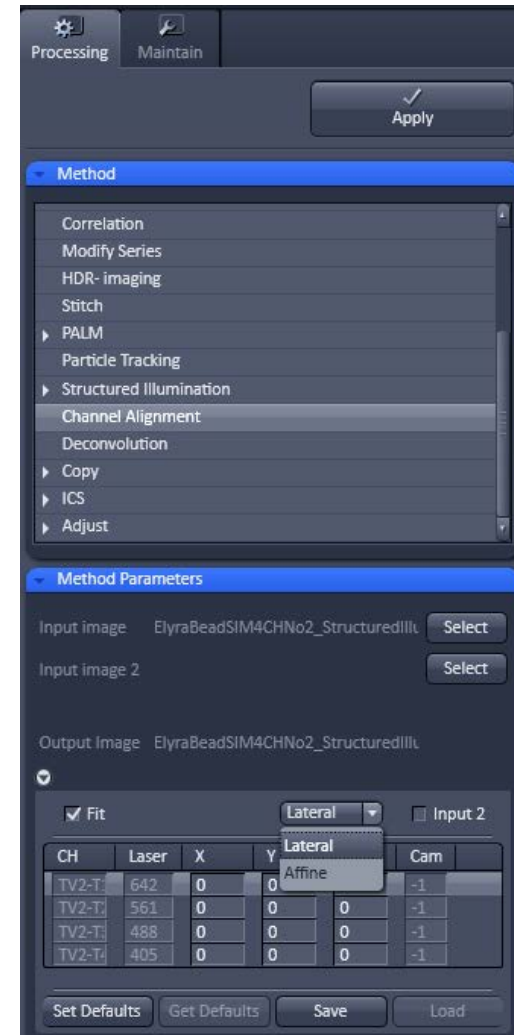
## 2. Combine the two “convert to image” files



2) Combine the two “convert to image” files into one image using the Channel Alignment function

- Select the two colors into “input 1” and “input 2”, check “input 2” box, uncheck “Fit” and apply.
- Note: the data should be simply combined and not shifted/corrected.
- Note: The file size of the combined image must be less than 4 GB. If not, reduce the number of pixels or size of image.

**Uncheck “Fit”**



**Select Ch 1 image**

**Select Ch 2 image**

**Check “Input 2”**

# 3D PALM Multicolor Channel Alignment

## 3. Calculate shifts and align channels

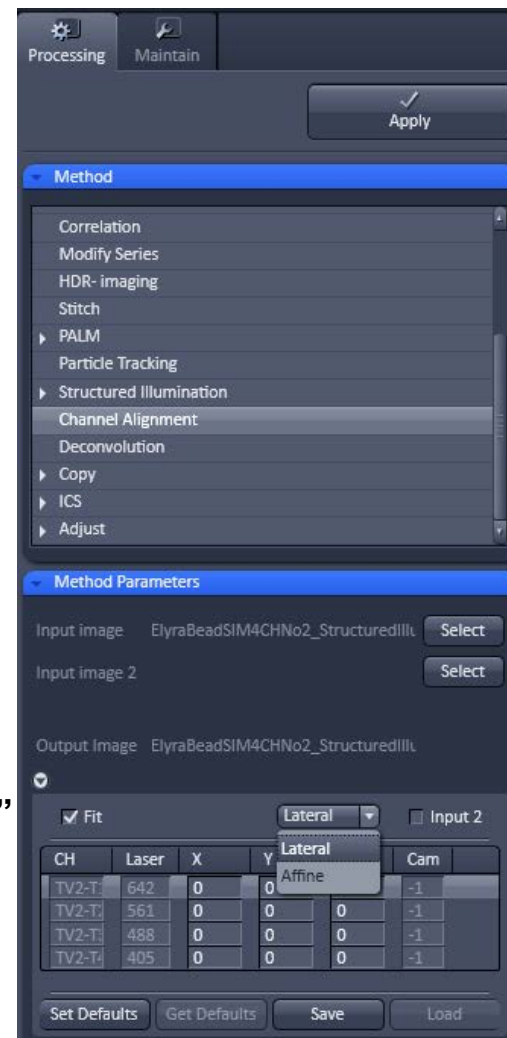


3) Use the Channel Alignment function again on the resulting image from step 2.

- a. Select the result from step 2 above into “input 1”
- b. Uncheck “input2”, uncheck “fit”
- c. Based on the x,y,z positions of the corrected fiducials you wrote in step **1b** above, calculate the average pixel shifts required for the channel alignment function:
  - i. Fiducial\_A = (Xa, Ya, Za), and Fiducial\_B = (Xb, Yb, Zb)
  - ii. Shift coefficient X =  $Xa - Xb / \text{pixel size}$
  - iii. Shift coefficient Y =  $Ya - Yb / \text{pixel size}$
  - iv. Shift coefficient Z =  $Za - Zb / \text{zSliceThickness}$

Uncheck “Fit”

Continued on next slide...



Select combined image with “Input 1”

Uncheck “Input 2”



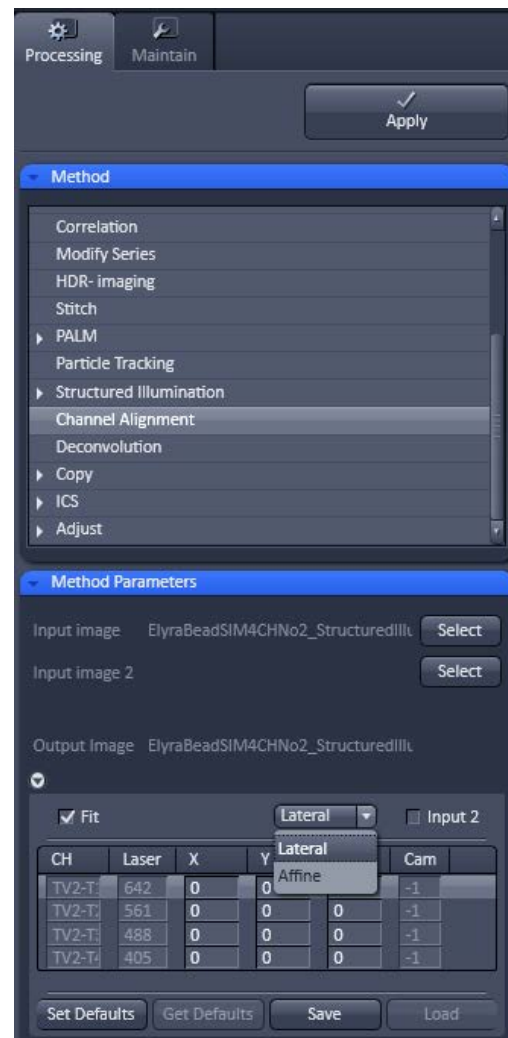
# 3D PALM Multicolor Channel Alignment



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  - i. Make sure the channels are properly matched ( ID = 0 is matched to first channel, ID = 1 matched to second channel)
- b. Hit Apply

Note 1: Consider the sign of the coefficients to make sure they are not flipped.

Note 2: The quality of the color fits depend critically on the quality of the fiducial drift correction. If you have poor fiducials or very few (2 or less), then the alignment may not be accurate.



Choose  
“Lateral”

Fill in desired  
x, y, z shifts



We make it visible.